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(54) DNA fragments having basidiomycete-derived promoter activity and their use

(57) This invention relates to a *Coriolus hirsutus* host cell transformed with a vector containing a basidiomycete-derived promoter region selected from the group consisting of basidiomycete-derived *ras* and *priA* gene promoter regions. This invention is particularly characterized in that the basidiomycete-derived promoter region is a *Lentinus edodes*-derived *ras* or *priA* gene promoter region or a *Coriolus hirsutus*-derived *ras* gene promoter region. This host cell can be utilized for the high productivity of lignin degrading enzymes.

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Description

BACKGROUND OF THE INVENTION

Field of the Invention

[0001] This invention relates to use of DNA fragments having promoter activity from *basidiomycetes,* particularly *Coriolus hirsutus* and *Lentinus edodes* in production of useful polypeptides such as lignin degrading enzymes. More specifically, this invention relates to a *Coriolus hirsutus* host cell transformed with a vector including a *Coriolus hirsutus*-derived *ras* promoter region or a *Lentinus edodes*-derived *ras* or *priA* promoter region, and to a method for producing useful polypeptides using the host cell.

[0002] According to this invention, polypeptides such as lignin degrading enzymes, the production of which has been considered to be difficult, can be supplied stably and in large quantity by genetic recombination technique using the host-vector system, particularly *basidiomycete*-derived host-vector system, of the present invention.

Background Art

[0003] Conventionally, wood pulps have been produced popularly by methods of chemically treating woods. However, from the viewpoint of environmental problems or the like, there has been an attempt to produce a cellulose pulp by inoculating a white rotting fungus into a lignocellulose substance of wood or the like then culturing the fungus to degrade lignin (Japanese Patent Application Laid-open No. 46,903/1975). However, the white rotting fungus used in this method has problems that the coexisting carbohydrates are degraded or, in cases of using cellulase-deficient mutants, their native lignin degrading ability becomes weaken, so that this method has not yet been put into practice.

[0004] In order to solve such problems as above, there has been an attempt to make a lignin degrading enzyme from a white rotting fungus act on a lignocellulose substance to selectively degrade lignin alone (*Science*, 221, 661 (1983)). This report, though mainly a lignin model compound is used as a substrate, is the first one in the world in which a lignin degrading enzyme was isolated and purified. This enzyme is an extracellular enzyme which *Phanerochaete chrysosporium* produces, and the main characteristics of the enzyme are as follows: the optimum pH is 3.0; it is an iron-containing enzyme; the molecular weight is 41,000 to 42,000 Da; hydrogen peroxide is necessary for enzymatic reaction; and it is confirmed to act on a compound formed by replacing the phenolic hydroxyl group at position 4 of a lignin model compound with a methoxyl group. This enzyme is lignin peroxidase, and the existence of plural isozymes are known (*FEBS Lett.*, 169, 247 (1984)). Lignin peroxidase is found from many wood-rotting fungi such as *Coriolus versicolor, Bjerkandera adusta*, etc. other than the above, some of which have been purified.

[0005] On the other hand, lignin degrading enzymes to be produced by *Coriolus hirsutus* and *Lenzites betulina* are also known (Japanese Patent Application Laid-open Nos. 220,190/1987 and 220,189/1987), which are phenol oxidases, and their main characteristics are that the optimum pH is 4.5, that they are copper-containing enzymes, that the molecular weight is approximately 63,000 Da or approximately 65,000 Da, that the isoelectric point is around 3.5, that oxygen is necessary for the enzymatic action, and that they do not act on a compound formed by replacing the phenolic hydroxyl group at position 4 of a lignin model compound with a methoxyl group but on a phenolic lignin model.

[0006] Besides, manganese peroxidase is one of typical lignin degrading enzymes, the main characteristics of which are that the molecular weight is 46,000 Da or less, that it is presumed to be an iron-containing enzyme, that hydrogen peroxide is necessary for the enzymatic action, that the enzyme reaction is Mn(II)-dependent, that it is confirmed to never act on a compound formed by replacing the phenolic hydroxyl group at position 4 of a lignin model compound with a methoxyl group but on a phenolic lignin model, and therefore it is an enzyme having properties quite different from those of the lignin peroxidase.

[0007] Up to now, the production of varieties of polypeptides by recombinant DNA techniques has been carried out using a host system centering around *Escherichia coli* (also designated *E. coli*). However, there are many cases where *E. coli* is not appropriate as a host. For example, there are many problems, in cases of producing useful polypeptides (e.g., enzymes) from a higher animal such as human being or the like, that the polypeptide of interest is not produced as an active protein, and that a large number of toxic substances other than the polypeptide of interest are produced making the purification of the target product very difficult. As alternative means for solving these problems, production methods using yeast, a lower eukaryote, as a host have been studied extensively, which however newly bring about a problem of low productivity. For the purpose of polypeptide production, transformation systems using higher eukaryotes such as filamentaous fungi (e.g. *Aspergillus*) and basidiomycetes (e.g. *Phanerochaete* and *Coriolus*) as hosts have also been developed, and the production of lignin degrading enzymes using the system has been studied.

[0008] Basidiomycetes belong to eukaryotes and are considered more closely related to animal cells than yeast (T. L. Smith, *Proc. Natl. Acad. Sci. USA*, 86, 7063 (1989)). *Coriolus hirsutus* having a strong lignin degrading ability is a basidiomycete belonging to the genus *Coriolus,* whose host-vector system has been developed by the present inven-

tors employing recombinant DNA techniques. As a result, the present inventors succeeded in the production of lignin peroxidase that had been considered to be difficult so far (Japanese Patent Application Laid-open No. 054,691/1994). However, the promoter region used therein was a promoter region of the ornithine carbamoyltransferase gene (hereinafter referred to as "OCT gene"), which is a gene for amino acid synthetase, or a promoter region of the phenol oxidase gene participating in lignin degradation, so there were problems that the productivity of the lignin peroxide was as low as that produced by a wild strain IFO4917 cultured in a lignin peroxidase production medium (low carbon and nitrogen sources), and that it took a lot of time for gene expression because the target enzyme was obtained as a secondary metabolite. In addition, it has been reported that a promoter, which is provided for enzyme protein production systems by genetic recombination of other organism species (e.g. filamentous fungi), was not function in basidiomycetes (A. Lorna et. al., *Curr. Genet.*, 16, 35 (1989)). Furthermore, ArgB gene from *Aspergillus nidulans* did not function in *Coriolus hirsutus* (A. Tsukamoto et al., USP 5,362,640).

[0009] Therefore, the present inventors obtained a promoter for constitutively expressing *Coriolus hirsutus* glyceraldehyde-3-phosphate dehydrogenase (GPD) gene, ligated thereto a structural gene with signal peptide coding sequence of a high temperature-induced lignin peroxidase gene (Japanese Patent Application Laid-open No. 260,978/1993) or a manganese per- oxidase gene (Japanese Patent Application Laid-open No. 308,581/1996) cloned from the *Coriolus hirsutus*, transformed the ligated product into an ornithine carbamoyltransferase-deficient *Coriolus hirsutus* mutant, and thereby succeeding in obtaining a strain capable of highly producing lignin peroxidase or manganese peroxidase (Japanese Patent Application Laid-open No. 47,289/1997).

[0010] However, in spite of the above technical proposal, a growing interest is taken in a promoter having a strong transcription activity and enabling high expression of a useful polypeptide.

[0011] Under such circumstances, the present inventors noticed a basidiomycete host-vector system and searched for various promoters functioning in this system.

[0012] Therefore, an object of the present invention is to provide a promoter for allowing a host such as basidiomycete or to produce a useful polypeptide in a large quantity.

[0013] Another object of the present invention is to provide a host-vector system including the promoter and a hyperexpression and production method of a useful polypeptide utilizing the system.

SUMMARY OF THE INVENTION

[0014] The present inventors noticed a constitutively expressing *Coriolus hirsutus ras* gene and *Lentinus edodes ras* and *priA* genes, the *priA* gene being expresses highly when a fruit body primoridium is formed in *Lentinus edodes*, cloned a DNA fragment encoding the *ras* gene or the *priA* gene from a *Coriolus hirsutus* or *Lentinus edodes* chromosomal DNA restriction fraction, sequenced a promoter region upstream of this gene, and found for the first time that this promoter region was effective for the expression of a gene encoding a useful polypeptide in a hostvector system, particularly *Coriolus hirsutus* host-vector system. Furthermore, the present inventors ligated a structural gene with signal peptide coding sequence of a manganese peroxidase gene, high temperature-induced lignin peroxidase gene (Japanese Patent Application Laid-open No. 260,978/1993), or laccase gene, the structural gene having been cloned from *Coriolus hirsutus*, to a site downstream of the above promoter region, introduced the same into an OCT-deficient *Coriolus hirsutus* mutant, and thereby succeeded in obtaining a manganese peroxidase-, lignin peroxidase- or laccase-highly producing strain with an ability to strongly degrade lignin.

[0015] That is, the present invention is summarized as follows.

(1) A *Coriolus hirsutus* host cell transformed with a vector containing a basidiomycete-derived promoter region selected from the group consisting of *ras* and *priA* gene promoter regions from basidiomycetes.
(2) A host cell defined in (1) above, wherein the *ras* gene promoter region is derived from *Coriolus hirsutus* or *Lentinus edodes.*
(3) A *Coriolus hirsutus* host cell defined in (1) above, wherein the *priA* gene promoter region is derived from *Lentinus edodes.*
(4) A *Coriolus hirsutus* host cell defined in (1) above, wherein the vector further comprises a gene encoding a useful polypeptide, the gene being transcribably ligated to a site downstream of the foregoing promoter region.
(5) A *Coriolus hirsutus* host cell defined in above (3), wherein the said gene encoding a useful polypeptide is a gene coding for a lignin degrading enzyme such as manganese peroxidase, lignin peroxidase, or laccase.
(6) A process for producing a useful polypeptide comprising culturing the *Coriolus hirsutus* host cell recited in (1) above in a medium and recovering the formed useful polypeptide.
(7) A process defined in (6) above, wherein the useful polypeptide is a lignin degrading enzyme such as manganese peroxidase, lignin peroxidase, or laccase.
(8) An isolated DNA fragment containing a *Coriolus hirsutus*-derived *ras* gene promoter region.
(9) An isolated DNA fragment defined in (8) above, wherein the DNA fragment has a nucleotide sequence shown

in SEQ ID NO:1 or a sequence that hybridizes to a sequence complementary to the nucleotide sequence under stringent conditions and has a promoter activity.

(10) A recombinant DNA, which contains a gene encoding a useful polypeptide and the DNA fragment recited in (8) above, the said gene being transcribably linked to the DNA fragment.

(11) A recombinant DNA defined in (10) above, wherein the gene encoding a useful polypeptide is a gene coding for a lignin degrading enzyme such as manganese peroxidase, lignin per-oxidase, or laccase.

(12) A DNA containing a *Coriolus hirsutus*-derived *ras* gene promoter sequence and *ras* gene sequence and has a nucleotide sequence shown in SEQ ID NO:2.

(13) A vector containing the DNA fragment recited in (8) above or the recombinant DNA in (10) above.

(14) A host cell transformed with the vector recited in (13) above.

(15) A host cell defined in (14) above, wherein the host is a basidiomycete.

(16) A host cell defined in (15) above, wherein the basidiomycete is *Coriolus hirsutus* .

(17) A process for producing a useful polypeptide, comprising culturing the host cell recited in (13) above in a medium and recovering the formed useful polypeptide.

(18) A process defined in (17) above, wherein the useful polypeptide is a lignin degrading enzyme such as manganese peroxidase, lignin peroxidase, or laccase.

BRIEF DESCRIPTION OF THE DRAWINGS

[0016]

Fig. 1 shows a plasmid pLC1MP containing a *Coriolus hirsutus*-derived manganese peroxidase gene whose expression regulation is governed by a *Lentinus edodes ras* gene promoter region.

Fig. 2 shows a plasmid pLC2MP containing a *Coriolus hirsutus*-derived manganese peroxidase gene whose expression regulation is governed by a *Lentinus edodes priA* gene promoter region.

Fig. 3 shows a plasmid pLC1LAC containing a *Coriolus hirsutus*-derived laccase gene whose expression regulation is governed by a *Lentinus edodes ras* gene promoter region.

Fig. 4 shows a plasmid pLC2LAC containing a *Coriolus hirsutus*-derived laccase gene whose expression regulation is governed by a *Lentinus edodes priA* gene promoter region.

Fig. 5 shows a chromosomal restriction map of a region containing *Coriolus hirsutus*-derived *ras* gene and promoter region thereof.

Fig. 6 shows the structure of the plasmid pCHRPRG for use in transformation of an arginine-requiring *Coriolus hirsutus* mutant illustrated in Example 11 below.

Fig. 7 shows the structure of the manganese peroxidase-expressing vector pCHRPMP illustrated in Example 13 below.

Fig. 8 shows the structure of the laccase-expressing vector pCHRPLAC illustrated in Example 16 below.

DETAILED DESCRIPTION OF THE INVENTION

[0017] Hereinafter the present invention will be described in detail.

[0018] In the first aspect, the present invention provides a DNA fragment containing a *Coriolus hirsutus*-derived *ras* promoter sequence.

[0019] The DNA fragment according to the present invention can be obtained through the following procedures.

[0020] Chromosomal DNA is prepared according to an ordinary chromosomal DNA extraction technique such as the method of Yelton et al. (*Proc. Natl. Acad. Sci. USA,* 81, 1470 (1984)) from *Coriolus hirsutus* (e.g., IFO4917 strain), and the obtained chromosomal DNA is treated with at least one appropriate restriction enzyme such as *Sau*3AI so as to partially degrade the DNA and then fractionated by sucrose gradient ultracentrifugation to obtain 10-kbp to 25-kbp DNA fragments. The DNA fragments obtained above are inserted into phage DNA treated with a restriction enzyme enabling the formation of the same cohesive end, thereby to generate a chromosomal gene library. As the phage DNA, EMBL3 (A-M, Frishauf et al., *J. Mol. Biol.*, 170, 827 (1983)) or λ phage DNA can be used. After insertion, packaging is carried out *in vitro* to give a chromosomal gene library. For subcloning, a plasmid such as pUC18 (C. Yanisch-Perron et al., *Gene*, 33, 103 (1985)) can be used. A cloning vector is not limited to those exemplified above, and commercially available ones or ones described in literature can also be used as the cloning vector. Next, from the obtained chromosomal gene library, a clone containing both a *ras* gene and a *ras* promoter region is selected by plaque hybridization using a synthetic DNA probe made on the basis of a nucleotide sequence of a *ras* gene isolated from other organism species. From the selected clone, a DNA fragment containing the target gene is isolated, of which a restriction map is made and a sequence is determined.

[0021] The sequencing can be carried out by inserting the above fragment containing *ras* chromosomal gene into

an appropriate cloning vector (e.g., a pUC vector such as pUC19) and then following the method of Sanger et al. (*Proc. Natl. Acad. Sci. USA*, 74, 5463 (1977)).

[0022] According to the above procedures, the restriction map shown in Fig. 5 and the nucleotide sequence shown in SEQ ID NO:2 were determined. A gene carrying this sequence is a 3497bp genomic gene containing a *Coriolus hirsutus*-derived *ras* gene and a *ras* gene promoter region upstream of the *ras* gene. In this sequence, the promoter region exists between nucleotide Nos. 1-1362 (SEQ ID NO:1), and TATAA at nucleotide Nos. 1045-1049 and CCAAA at nucleotide Nos. 977-981 are recognized respectively. On the other hand, the *ras* gene exists between Nucleotide Nos. 1363-2419 and is composed of 7 exons and 6 introns (intervening sequences). Specifically, the exons and introns exist as follows respectively; Exon 1 exists between 1363-1371, Intron 1 between 1372-1425, Exon 2 between 1426-1465, Intron 2 between 1466-1517, Exon 3 between 1518-1592, Intron 3 between 1593-1717, Exon 4 between 1718-1800, Intron 4 between 1801-1861, Exon 5 between 1862-2055, Intron 5 between 2056-2113, Exon 6 2114-2240, Intron 6 between 2241-2296, and Exon 7 between 2297-2419. Besides, the region between nucleotide Nos. 2420-3497 is a 3' non-translational region containing a terminator. From the sequence analysis, *Coriolus hirsutus ras* protein was found to have the amino acid sequence given in SEQ ID NO:3 with 213 amino acids.

[0023] *Escherichia coli* strain DH5α/pCHRAS carrying a genomic gene fragment containing *Coriolus hirsutus*-derived *ras* gene/promoter sequence (SEQ ID NO:2) was deposited in National Institute of Bioscience & Human-Technology, Agency of Industrial Science & Technology (Higashi 1-1-3, Tsukuba-shi, Ibaraki-ken, 305-8566 Japan) on March 30, 1999 and was given the Accession No. FERM P-17352, which deposit was transferred to the international deposit on January 20, 2000 in accordance with the terms of the Budapest Treaty and given the Accession No. FERM BP-7001. The DNA having the nucleotide sequence shown in SEQ ID NO:2 contained in this deposited strain is included within the scope of the present invention.

[0024] A DNA fragment containing the *ras* gene promoter region can be obtained from the above fragment containing *ras* chromosomal gene by PCR (polymerase chain reaction). As primers for PCR, sequences with approximately 10 to 50 nucleotides, preferably approximately 15 to 30 nucleotides based on the nucleotide sequence shown in SEQ ID NO:1 and their complementary sequences can be used as sense and antisense primers. For example, the sense and antisense primers shown in SEQ ID NOS:5 and 6, respectively, can be used (see Example 11 below).

[0025] As used herein, the term "Promoter sequence" or "promoter region" refers to a sequence or region having a function to regulate the transcription of a structural gene, and it at least contains a functional nucleotide sequence motif that is substantially conserved in eukaryotic promoters (e.g., TATA, CCAA, and GC boxes). Therefore, the nucleotide sequence given in SEQ ID NO:1 is an specific example of such promoter sequences, and a sequence hybridizing to a sequence complementary to the said nucleotide sequence under stringent conditions or a sequence containing mutation or modification such as deletion, substitution or addition of one or more nucleotides in the said nucleotide sequence also falls in the scope of the present invention. Such mutation or modification can be conducted by employing a well-known site-specific mutagenesis technique such as oligonucleotide site-specific mutagenesis, or cassette mutagenesis, based on the nucleotide sequence given in SEQ ID NO:1 (e.g., see Short Protocols In Molecular Biology, Third Edition, John Wiley & Sons, Inc.).

[0026] As used herein, the term "Stringent Conditions" means conditions where it is possible to hybridize to a mutated or modified sequence having 70% or higher, preferably 80% or higher, more preferably 90% or higher, particularly 95% or higher homology to the complementary strand of the nucleotide sequence shown in SEQ ID NO:1. Generally, hybridization conditions are determined in consideration of factors such as temperature, ion intensity and the like, and it is known that stringency generally becomes higher as the temperature becomes higher and/or as the ion intensity becomes lower. Specific conditions are an ion intensity of 6×SSC and a hybridization temperature of 68°C.

[0027] In the second aspect, the present invention provides a recombinant DNA which contains both a gene encoding a useful polypeptide and a DNA fragment containing *Coriolus hirsutus*-derived *ras* gene promoter sequence, the said gene being transcribably linked to the said DNA fragment.

[0028] As used herein, the term "transcribably" means that the transcription of the above gene into mRNA takes place under the action of a promoter in a host. A gene encoding a useful polypeptide is linked at a site downstream of a DNA fragment containing a promoter sequence and transcribed into mRNA by action of the promoter. As a gene encoding a useful polypeptide, any non-limited gene can be used. Examples thereof include genes for lignin degrading enzymes such as manganese peroxidase, lignin peroxidase, laccase, etc. These genes are obtainable according to the well-known genome or cDNA cloning techniques and PCR techniques using sequences registered in gene banks or sequences described in literature. Alternatively, in cases of deposited genes, genes available by request for furnished samples can be used. The DNA fragment containing a promoter sequence and the gene encoding a useful polypeptide can be ligated together using an appropriate DNA ligase after, optionally, introducing a restriction site and making blunt-ends or cohesive ends. As recombinant DNA techniques including cloning, ligation, PCR, etc., those described, for example, in J. Sambrook et al., Molecular Cloning, A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory press, 1989 and Short Protocols In Molecular Biology, Third Edition, A Compendium of Methods from Current Protocols in Molecular Biology, John Wiley & Sons, Inc. can be used.

[0029] In the third aspect, the present invention provides a vector containing the DNA fragment or recombinant DNA defined above.

[0030] A vector, but not limited to a specific type of vector, is selected depending on the kind of a host to be transformed with the vector. Used as the vectors are ones autonomously replicable in a prokaryotic or eukaryotic host cell or ones homologously integrated in the chromosome, including plasmids, viruses, phages, cosmids, etc. The vector can optionally include a selective marker, an origin of replication, a terminator, a polylinker, an enhancer, a ribosome binding site, etc. Since various vectors for prokaryotes and eukaryotes such as bacteria, fungi, yeasts, animals and plants are commercially available or are described in literature, the DNA fragment or recombinant DNA of the invention can be inserted into the aforementioned vectors. The insertion of the DNA can be carried out by utilizing known techniques such as those described in J. Sambrook et al. (*ibid*).

[0031] In the fourth aspect, the present invention further provides a host cell transformed with the vector prescribed above.

[0032] Here, any host cell can be selected from fungi including basidiomycetes, filamentous fungi, and yeasts, other eukaryotic cells (e.g., animal cells, plant cells, insect cells and algae) and prokaryotic cells (e.g., bacteria and blue-green algae) as far as a structural gene of interest is expressed under the control of the promoter. A preferable host cell is *Coriolus hirsutus*, which is a class of basidiomycetes. Methods of transformation include, but are not limited to, calcium chloride/PEG method, calcium phosphate method, lithium acetate method, electroporation method, protoplast method, spheroplast method, lipofection method, and agrobacterium method. In examples described later, an expression example using *Coriolus hirsutus* as a host is given, in which the ornithine carbamoyltransferase (OCT) -deficient *Coriolus hirsutus* auxotrophic mutant OJI-1078 (Accession No. FERM BP-4210) is used as a host.

[0033] In the fifth aspect, the present invention provides a process for producing a useful polypeptide comprising culturing the above transformed host cell in a medium and recovering the produced useful polypeptide.

[0034] A polypeptide is produced in a secretory form when it is expressed and translated in a fusion form with a signal peptide, and in this case the polypeptide can directly be isolated from a medium. On the other hand, when a polypeptide is produced in a non-secretory form, cells are isolated and then destroyed by treatments such as ultrasonication or homogenization to obtain an extract, from which the polypeptide can be isolated. Isolation and purification can be carried out by adopting methods such as solvent extraction, salting out, desalting, organic solvent precipitation, ultrafiltration, ion exchange, hydrophobic interaction, HPLC, gel filtration, affinity chromatography, electrophoresis, chromatofocusing, etc., which are used alone or in combination.

[0035] For example, when the structural gene portion for manganese peroxidase, lignin peroxidase or laccase is ligated at a site downstream of the promoter region of the invention and recombinant DNA techniques are employed, the manganese peroxidase, lignin peroxidase or laccase can be produced in a large quantity. An *Escherichia coli* recombinant strain JM109/pBSMPOC1 containing a *Coriolus hirsutus*-derived manganese peroxidase cDNA (Japanese Patent Application Laid-Open No. 308581/1996), an *Escherichia coli* recombinant strain JM109/pBSMPOG1 containing a manganese peroxidase chromosomal gene (Japanese Patent Application Laid-Open No. 308581/1996), an *Escherichia coli* recombinant strain *E. coli* XL-1 blue/pBSLPOG7 containing a high temperature-induced lignin peroxidase gene (Japanese Patent Application Laid-Open No. 260978/1993), and an *Escherichia coli* recombinant strain containing a laccase (also named phenol oxidase) gene (Japanese Patent Publication Nos. 46,995/1995 and 85,717/1994) have been deposited in the National Institute of Bioscience & Human-Technology, Agency of Industrial Science & Technology (Higashi 1-1-3, Tsukuba-shi, Ibaraki-ken, Japan) under Accession Nos. FERM P-14932, FERM P-14933, FERM P-12683, and FERM BP-2793 (chromosomal gene), FERM P-10055 (cDNA) or FERM P-10061 (cDNA), respectively.

[0036] In the sixth aspect, the present invention provides a *Coriolus hirsutus* host cell transformed with a vector containing a basidiomycete-derived promoter region selected from the group consisting of basidiomycete-derived *ras* gene promoter and *priA* gene promoter regions.

[0037] In one embodiment of the present invention, the *ras* gene promoter is derived from *Lentinus edodes* or *Coriolus hirsutus* , and the *priA* gene promoter from *Lentinus edodes*. *Coriolus hirsutus ras* gene promoter region can be obtained as above. Besides, the *Lentinus edodes*-derived *ras* gene promoter and *priA* gene promoter regions can be obtained from *Lentinus edodes* (e.g., MAFF-430002 available from Bio-Related Industrial Technology and Research Advancing Organization (http://www.brain.go.jp); MAFF-430002 strain) in accordance with the method described in *FEMS Microbiology Letters*, 92, 147 (1992) and *Gene*, 114, 173 (1992), respectively.

[0038] In the present invention, when a gene encoding a useful polypeptide is transcribably linked at a site downstream of the above promoter region in the above-described vector, a *Coriolus hirsutus* transformant obtained by transformation with the vector can produce the polypeptide in a high yield by culturing the transformant in an appropriate medium. Examples of such useful polypeptides include lignin degrading enzymes such as manganese peroxidase, lignin peroxidase and laccase.

[0039] As described above, basidiomycetes producing lignin degrading enzymes such as manganese peroxidase, lignin per- oxidase and laccase, particularly including a *Coriolus hirsutus* transformant having an ability to highly degrade lignin, are provided according to the present invention. The transformant of the present invention has a prop-

erty of acting on lignin so as to degrade it into low-molecular-weight products, so it can be applied to various steps in paper and pulp production processes using a lignocellulose material of wood or the like as a raw material. Besides, in saccarification of wood, this transformant can also be applied to the field of a so-called cellulose biomass utilization for increasing the cellulase action by decomposing lignin as a treatment at the preceding stage of saccarification.

EXAMPLES

[0040] Hereinafter, the present invention will be described in further detail with examples. However, the scope of the pre- sent invention is not limited by the examples.

Example 1

Construction of Expression Vector Containing *Coriolus hirsutus*-Derived Manganese Peroxidase Gene with *Lentinus edodes*-Derived *ras* Gene Promoter

[0041] A plasmid pLC1 (6.4kb) containing a *Lentinus edodes*-derived *ras* gene promoter region (2.5kb) and a *priA* gene terminator region (1.2kb) (K. Ogawa et al., Appl. Microbiol. Biotechnol. (1998) 49, 285-289) was digested with a restriction enzyme *Bam*HI (Takara Shuzo Co., Ltd., Kyoto, Japan), followed by ligating a *Coriolus hirsutus*-derived manganese peroxidase cDNA to the *Bam*HI site in the forward direction to prepare a manganese peroxidase gene expression vector, which was designated as pLC1MP (Fig. 1).

Example 2

Construction of Expression Vector Containing *Coriolus hirsutus*-Derived Manganese Peroxidase Gene with *Lentinus edodes*-Derived *priA* Gene Promoter

[0042] A plasmid pLC2 (5.6kb) containing a *Lentinus edodes*-derived *priA* gene promoter region (0.4kb) and a *priA* gene terminator region (1.2kb) (K. Ogawa et al., *ibid*) was digested with a restriction enzyme *Bam*HI (Takara Shuzo Co., Ltd., Kyoto, Japan), followed by ligating a *Coriolus hirsutus*-derived manganese peroxidase cDNA gene to the *Bam*HI site in the forward direction to prepare a manganese peroxidase gene expression vector, which was designated as pLC2MP (Fig. 2).

Example 3

Method for Transforming *Coriolus hirsutus*

[0043] The following procedures (a) to (c) were carried out, and a transformant was obtained by the following (d) and (e).

(a) Mononuclear hypha culture

[0044] 100ml of SMY medium (1% sucrose, 1% malt extract, 0.4% yeast extract) was dispensed in a 500-mL conical flask, in which approximately 30 glass beads (around 6mm diameter) were placed, and sterilized. Then, agar piece (5 mm diameter) was punched out from a plate agar culture of *Coriolus hirsutus* strain OJI-1078 using a cork borer, which was then inoculated into a SMY medium and stationarily cultured at 28°C for 7 days (preculture).

[0045] To fractionize the hypha, the medium was shaken once or twice a day. Next, 200ml of the SMY medium was dispensed into a 1-L conical flask, in which a rotator was placed. After sterilization, the precultured hypha was filtered and collected with a nylon mesh (pore size, 30µm) and stationarily cultured at 28°C. In this step, the hypha was fractionized by stirring with a stirrer for 2 hours a day. This culture was carried out for 4 days.

(b) Preparation of protoplast

[0046] The above liquid-cultured hypha was filtered and collected with a nylon mesh (pore size, 30µm), followed by washing with an osmotic pressure regulating solution (0.5M $MgSO_4$, 50mM maleate buffer (pH5.6)). Next, the wet hypha (100mg/ml) was suspended in a cell wall lysing enzyme solution and incubated at 28°C for 4 hours with gently shaking to make protoplasts free. As the cell wall digesting enzyme, the following commercially available enzyme preparations were used in combination. That is, 5mg of cellulase Onozuka RS (Yakult Co., Ltd.,Tokyo, Japan) and 10mg of Yatalase (Takara Shuzo, Co., Ltd., Kyoto, Japan) were dissolved in 1ml of the above osmotic pressure regulating solu-

tion, which was used as an enzyme solution.

(c) Purification of protoplast

[0047] After removing the fractionized hypha from the above enzymatic reaction mixture with a nylon mesh (pore size, 30 μ m), the fractionized hypha and protoplasts remaining on the nylon mesh were washed once with the above osmotic pressure regulating solution in order to increase the recovery of protoplast. The obtained protoplast suspension was centrifuged (1,000xg, 5 minutes), followed by removing the supernatant. After resuspending the precipitate in 4ml of 1M sucrose solution (20mM MOPS buffer (pH 6.4)), centrifugation was repeated and the resulting precipitate was washed twice with the above 1M sucrose solution. Thus treated precipitate was suspended in 500 μl of a solution prepared by adding 40mM calcium chloride to 1M sorbitol solution (20mM MES (pH 6.4)) to prepare a protoplast suspension, which was preserved at 4°C.

[0048] The protoplast concentration was determined by direct microscopic observation using a hemocytometer. The centrifugation steps were all carried out using a swing rotor at 1,000$^{\times}$g at room temperature for 5 minutes.

(d) Transformation-1

[0049] To 100 μl of the protoplast suspension with a concentration of 10^6 cells/100 μl, 10 μg of plasmid pLC1MP prepared in Example 1 and 1 μg of pUCR1 containing an ornithine carbamoyltransferase chromosomal gene were added at the same time, followed by ice-cooling the mixture for 30 minutes. Then, an equal volume of a PEG solution (50% PEG3400, 20mM MES (pH 6.4)) was added to the protoplast suspension, followed by ice-cooling for 30 minutes. Next, the ice-cooled mixture was mixed in a mini- mum soft agar medium (1% agar) containing 0.5M sucrose and leucine and inoculated onto a plate. The plate was cultured at 28°C for 4 days, whereby a transformant was obtained.

(e) Transformation-2

[0050] To 100 μl of the protoplast suspension with a concentration of 10^6 cells/100 μl, 10 μg of plasmid pLC2MP prepared in Example 2 and 1 μ g of pUCR1 containing an ornithine carbamoyltransferase chromosomal gene were added at the same time, followed by ice-cooling the mixture for 30 minutes. Then, an equal volume of a PEG solution (50% PEG3400, 20mM MES (pH 6.4)) was added to the protoplastsuspension, followed by ice-cooling for 30 minutes. Next, the ice-cooled mixture was mixed with a mini- mum soft agar medium (1% agar) containing 0.5M sucrose and leucine and inoculated onto a plate. The plate was cultured at 28°C for 4 days, whereby a transformant was obtained.

Example 4

Decloration and Decomposition of Lignin with Transformant

[0051] The transformant obtained in Example 3 was inoculated in 10ml of MYG medium (1% malt extract, 0.4% yeast extract, 0.4% glucose; pH 5.6) containing 10mg of lignin (Tokyo Kasei Kogyo K.K., Tokyo, Japan) and 2.5mg of $MnCl_2$ and subjected to shake culture at 30°C. Separately, a host strain OJI-1078 was inoculated into the same medium and similarly subjected to shake culture as a control. The degradation of lignin was measured serving as an indication the decrease in absorbance at 275nm indicating cleavage of aromatic rings. The decoloration of lignin was measured serving as an indication the decrease in absorbance at 480nm. As the result, the absorbance of 10ml of MYG liquid medium containing 10mg of lignin was 27.15 at 275nm and 2.19 at 480nm. These values at the start of culture were defined as 100%.

[0052] As the result, in the case of the transformant for manganese peroxidase expression using the *ras* gene promoter region, the degradation of lignin and decoloration was increased about 3 times as compared with the host as a control 16 days after the start of culture.

[0053] The transformant for manganese peroxidase expression using the *priA* gene promoter region showed about 4 times higher lignin degradation and decoloration than the case of only the host 12 days after the start of culture.

Example 5

Production of Manganese Peroxidase by Transformant

[0054] Five 50-mm^2 agar pieces containing each of the transformed strains obtained in Example 3 above were inoculated into 50ml of glucose-peptone liquid medium (20g/l glucose, 5g/l peptone, 2g/l yeast extract, 0.5g/l $KH_2PO_4 \cdot 7H_2O$, 48 mg/l $MnSO_4 \cdot 5H_2O$; adjusted to pH 5.0 with phosphoric acid) in a 500-mL conical flask and cul-

tured at 28°C for 6 days with shaking. After 6 days, the obtained culture was centrifuged to obtain the supernatant.

[0055] The enzyme activity was measured by thoroughly mixing 50 μl of 0.5M sodium malonate buffer (pH 5.5), 345 μl of enzyme solution, 5 μl of 10mM hydrogen peroxide and 100 μl of 1mM $MnSO_4$ and recording the increase in 270-nm absorbance of Mn(III)-malonic acid complex generated by the reaction over time. In the case of pLC1MC, the Mn(III)-malonic acid complex was observed in the above supernatant at a level of enzyme activity of 5μmol/ml/min 6 days after the start of culture. In the case of pLC2MP the complex was at 4μ mol/ml/min. Here, one unit of the enzyme activity is defined as an activity increasing 1μmol of the Mn(III)-malonic acid complex over 1 minute. On the other hand, this activity was not recognized in the supernatant of the target DNA-free strain OJI-1078 cultured under the same conditions.

Example 6

Construction of Expression Vector Containing *Coriolus hirsutus*-Derived Laccase Gene with *Lentinus edodes*-Derived *ras* Gene Promoter

[0056] A plasmid pLC1 (6.4kb) containing a *Lentinus edodes*-derived *ras* gene promoter region (2.5kb) and a *priA* gene terminator region (1.2kb) was digested with a restriction enzyme *Bam*HI (Takara Shuzo Co., Ltd., Kyoto, Japan), followed by ligating a *Coriolus hirsutus*-derived laccase cDNA gene (Accession No. FERM P-10055) to the *Bam*HI site in the forward direction to prepare a laccase gene expression vector, which was designated as pLC1LAC (Fig. 3).

Example 7

Construction of Expression Vector containing *Coriolus hirsutus*-Derived Laccase Gene with *Lentinus edodes*-Derived *priA* Gene Promoter

[0057] A plasmid pLC2 (5.6kb) containing a *Lentinus edodes*-derived *priA* gene promoter region (0.4kb) and a *priA* gene terminator region (1.2kb) was digested with a restriction enzyme *Bam*HI (Takara Shuzo Co., Ltd., Kyoto, Japan), followed by ligating *Coriolus hirsutus*-derived laccase cDNA gene (Accession No. FERM P-10055) to the *Bam*HI site in the forward direction to prepare a laccase gene expression vector, which was designated as pLC2LAC (Fig. 4).

Example 8

Culture of Highly Laccase-Producible Strain

[0058] Each of the laccase gene expression vectors obtained in Examples 6 and 7 according to the method described in Example 3 was introduced into a host cell of *Coriolus hirsutus* strain 1078. Five 50-mm^2 agar pieces of each transformant was inoculated into 50ml of a glucose-peptone liquid medium (30g/l glucose, 10g/l peptone, 1.5g/l KH_2PO_4, 0.5g/l $MgSO_4 \cdot 7H_2O$, 100 mg/l $CuSO_4 \cdot 5H_2O$; adjusted to pH 5.0 with phosphoric acid) in a 500-mL conical flask and cultured at 28°C for 6 days with shaking. Six days later, the obtained culture was centrifuged to obtain the supernatant.

[0059] The enzyme activity was measured by mixing 50 μl of 1M sodium acetate buffer (pH 4.0), 50 μ l of 5mM 2,2'-azino-bis(3-ethilbenzthiazoline-6-sulfonate) (ABTS)and 400 μ l of enzyme solution and recording the increase in 420-nm absorbance of ABTS oxide generated by the reaction over time. In the case of pLC1LAC, the enzyme activity in the above supernatant was 34 units/ml on the 5th day after the start of culture. In the case of pLC2LAC, it was 26 units/ml. Here, one unit of the enzyme activity is defined as the quantity of enzyme requiring to oxidize 1μmol of ABTS over 1 minute. On the other hand, the activity recognized in the supernatant of the target DNA-free strain OJI-1078 cultured under the same conditions was only 5 units/ml.

Example 9

Preparation of Chromosomal Gene Library

[0060] Agar piece (5 mm diameter) was punched out from a plate agar culture of *Coriolus hirsutus* (IFO4917 strain available from the IFO, Osaka, Japan) using a cork borer, inoculated into 200ml of a glucose-peptone liquid medium (2% glucose, 0.5% polypeptone, 0.2% yeast extract, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$; adjusted to pH 4.5 with phosphoric acid), and subjected to rotary shaking culture at 28°C for 7 days. Then, the cell culture was harvested, washed with 1 liter of sterile water and then frozen with liquid nitrogen. Five g of this frozen cells were pulverized using a motor. After transferring the pulverized cells to a centrifugal tube, 10ml of abacterium-lysis buffer (100mM tris (pH8),

100mM EDTA, 100mM NaCl; further supplemented with Proteinase K at a concentration of 100 μg/ml) was added and the mixture was incubated at 55°C for 3 hours. The incubated mixture was treated with phenol and then with chloroform. Subsequently, ethanol was gradually added to the aqueous phase until DNA was separated out, and the separated chromosomal DNA was wound up and suspended in a TE solution.

[0061] 100 μg of the obtained chromosomal DNA was partially digested with a restriction enzyme *Sau*3AI, and fractionated by 5% to 20% sucrose gradient centrifugation (30,000rpm, 18 hours) to pool 20-40 kbp fragments. These fragments were ligated to phage λ EMBL3-*Bam*HI arm (Toyobo, Co., Ltd., Kyoto, Japan) with T4DNA ligase. After packaging the obtained phage DNA using a GIGAPACK GOLD (STRATAGENE, USA), it was infected with *Escherichia coli* strain P2392 to prepare a chromosomal DNA library.

Example 10

Isolation of *ras* Gene from Chromosomal Gene Library

[0062] The selection of a clone containing an ornithine carbamoyltransferase gene from the above chromosomal DNA library was carried out by plaque hybridization. This series of procedures were carried out according to ordinary methods such as those described in Sambrook et al., "Molecular Cloning" A Laboratory Manual/2nd Edition (1989).

[0063] The probe used in the plaque hybridization was prepared by radiolabeling synthetic oligomers having the following sequence with ^{32}P:

SEQ ID NO:4: 5'-CA(T/C)TT(T/C)GIGA(T/C)GA(A/G)TA(T/C)GA-3'

[0064] As a result, 4 positive clones could be selected from approximately 40,000 plaques. Recombinant phage DNAs prepared from the positive clones according to an ordinary method were digested with various restriction enzymes, and Southern Hybridization was carried out using the above synthetic DNAs. As a result, a clone hybridizing to a single 3.5 kbp DNA band was found in fragments obtained by digestion with a restriction enzyme *Bam*HI.

[0065] A 3.5-kbp DNA fragment was cut out of a gel following agarose gel electrophoresis, subcloned into the *Bam*HI site of *Escherichia coli* vector pUC19, and transformed into *Escherichia coli* strain JM109. The subcloned DNA was prepared in a large quantity, purified by centrifugation (50,000rpm, 16hrs, 15°C) and determined for its nucleotide sequence. The sequencing was carried out using a sequenase kit (United States Biochemical, Inc., USA).

[0066] The nucleotide sequence is given in SEQ ID NO:2. As seen in this sequence, the *Coriolus hirsutus*-derived *ras* gene is discontinued by 6 introns in the above nucelotide sequence. Furthermore, the amino acid sequence deduced from the nucleotide sequence was found to have a high homology to those of *ras* genes which had previously been reported. The amino acid sequence is given in SEQ ID NO:3.

[0067] The obtained *Escherichia coli* strain *E. coli* DH5 α /pCHRAS containing *Coriolus hirsutus ras* gene was deposited under the terms of the Budapest Treaty at the National Institute of Bioscience & Human-Technology, Agency of Industrial Science & Technology (Higashi 1-1-3, Tsukuba-shi, Ibaraki-ken, Japan) with Accession No. FERM BP-7001 (which was the one transferred to the international deposit on January 20, 2000 from FERM P-17352 deposited on March 30, 1999).

Example 11

Ligation of OCT Structural Gene Governed by *ras* Gene Control Region

[0068] A selective marker plasmid pCHRPRG for the expression of OCT of a basidiomycete *Coriolus hirsutus* was a selective marker prepared by ligating the structural gene region of the OCT chromosomal gene (Japanese Patent Application Laid-open No. 054691/1994; FERM BP-4201) to a site downstream of *ras* gene promoter region thereby to substitute the *ras* gene promoter region for the promoter region of the native OCT gene.

[0069] From a plasmid pCHRAS containing *Coriolus hirsutus*-derived *ras* gene promoter region, a DNA fragment containing the *Coriolus hirsutus*-derived *ras* gene promoter region was amplified by PCR method using the following 2 primers:

Primer-1: 5'-GGATCCCGCTATACCGAAAGG-3' (SEQ ID NO:5)
Primer-2: 5'-CCATGGCTGTATGGCGGAGG-3' (SEQ ID NO:6)

to give a 1.4 kbp BamHI-NcoI fragment which was then ligated to a unique SmaI site of pUC18 using T4 DNA ligase to obtain a plasmid pCHRP.

[0070] On the other hand, in order to isolate a gene region encoding a matured enzyme of OCT, the plasmid

pUCR1 (Japanese Patent Application Laid-open No. 054691/1994; FERM BP-4201) was digested with a restriction enzyme *NcoI* to obtain approximately 2.5-kbp DNA fragment.

[0071] The foregoing 2 kinds of DNA fragments were mixed, ligated together using T4DNA ligase, and transformed into *Escherichia coli* strain JM109. A plasmid into which the OCT gene had been inserted in the forward direction was isolated from the ampicillin-resistant transformant strains and was designated as pCHRPRG (Fig. 6).

Example 12

Transformation of *Coriolus hirsutus*

[0072] To 100 µl of a protoplast suspension having a concentration of 10^6/100 µl obtained in the same manner as in (a) to (c) of Example 3, 2µg of the plasmid pCHRPRG prepared in Example 11 was added and ice-cooled for 30 minutes. Then, an equal volume of a PEG solution (50% PEG3400, 20mM MES (pH 6.4)) was added to the above solution, followed by ice-cooling for 30 minutes. Next, the ice-cooled mixture was mixed with a minimum soft agar medium (1% agar) containing 0.5M sucrose and leucine and inoculated onto a plate. The plate was cultured at 28°C for several days to obtain transformants. The transformation efficiency was 300 colonies/ µg of transformed DNA. In contrast, in the control experiment using a DNA consisting of the plasmid vector pUC18 alone, any transformant was not obtained at all.

Example 13

Construction of Expression Vector Containing *Coriolus hirsutus*-Derived Manganese Peroxidase Gene with *Coriolus hirsutus*-Derived *ras* Gene Promoter

[0073] From a plasmid pCHRAS containing a *Coriolus hirsutus*-derived *ras* gene promoter region, the promoter region was amplified by PCR method using the following 2 primers:

Primer-1: 5'-GGATCCCGCTATACCGAAAG-3' (SEQ ID NO:5)
Primer-2: 5'-CCATGGCTGTATGGCGGAGG-3' (SEQ ID NO:6)

to give a 1.4 kbp BamHI-NcoI fragment which was then ligated to a unique *SmaI* site of pUC18 with T4DNA ligase to obtain a plasmid pCHRP.

[0074] The obtained plasmid pCHRP was digested with restriction enzymes *NcoI* and *Eco*RI (Takara Shuzo Co., Ltd., Kyoto, Japan) and then served as an expression vector.

[0075] Next, an approximately 2.2 kb *NcoI-EcoI* fragment, which was a manganese peroxidase structural gene portion of a plasmid pBSMPOG1 (Accession No. FERM P-14933) containing a *Coriolus hirsutus*-derived manganese peroxidase gene, was amplified by PCR method using the following 2 primers:

Primer-1: 5'-CCATGGCTTTCAAGACACTCG-3' (SEQ ID NO:7)
Primer-2: 5'-GAATTCGCATGTAGGTCCGCG-3' (SEQ ID NO:8)

[0076] The obtained PCR fragment was inserted into the plasmid using TA cloning kit (In Vitrogen, Inc.) to prepare pTAMP. Then, the obtained pTAMP was digested with restriction enzymes *NcoI* and *Eco*RI to obtain approximately 2.2 kb fragment which was inserted into the *NcoI-Eco*RI sites of the pCHRP to obtain a plasmid pCHRPMP (Fig. 7).

Example 14

Preparation of *Coriolus hirsutus* Transformant Highly Secreting and Producing Manganese Peroxidase

[0077] In transforming an arginine-requiring *Coriolus hirsutus* (OJI-1078 strain) with the pCHRPMP obtained in Example 13, a transformant pCHRPMP/OJI-1078 was obtained by simultaneously introducing a *Coriolus hirsutus*-derived OCT gene-carrying plasmid (pUCR1) as a selective marker (according to PEG method, electroporation or the like). Here, whether a DNA which can be subjected to transformation is in a circular or linear form, this transformation method could provide a transformant of interest. The conditions of transformation are as follows.

[0078] Two µg of the plasmid prepared in Example 13 was added to 100 µ l of the protoplast suspension having a concentration of approximately 10^6 cells/100 µl in a circular or linear form, followed by addition of 0.2 µg of pUCR1 as a selective marker then 30-minute ice-cooling.

[0079] Next, an equal volume of a PEG solution (50% PEG3400, 20mM MES (pH 6.4)) was added and the mixture was ice-cooled for 30 min. The ice-cooled mixture was mixed with a minimum soft agar medium (1% agar) containing

0.5M sucrose and leucine and inoculated onto a plate. The plate was cultured at 28°C for 4 days, whereby a transformant was obtained. From this transformed strain, a DNA is prepared, which was subjected to Southern hybridization in order to confirm that the manganese peroxidase-expressing plasmid of interest had been introduced into the strain.

Example 15

Production of Manganese Peroxidase by Transformant

[0080] Five 50-mm^2 agar pieces containing the transformant strain obtained in Example 14 were inoculated into 50ml of a glucose-peptone liquid medium (30g/l glucose, 10g/l peptone, 1.5g/l KH_2PO_4, 0.5g/l $MgSO_4 \cdot 7H_2O$, 2mg/l thiamine HCl, 48mg/l $MnSO_4 \cdot 5H_2O$; adjusted to pH 5.0 with phosphoric acid) in a 500-mL conical flask and cultured at 28°C for 6 days with shaking. After 6 days, the obtained culture solution was centrifuged to obtain the supernatant.

[0081] The enzyme activity was measured by well mixing 50 µl of 0.5M sodium malonate buffer (pH 5.5), 345 µl of enzyme solution, 5 µl of 10mM hydrogen peroxide and 100 µl of 1mM $MnSO_4$ and recording the increase in 270-nm absorbance of Mn(III)-malonic acid complex generated by the reaction over time. In the above supernatant, the Mn(III)-malonic acid complex was observed at a level of enzyme activity of 6 µmol/ml/min 6 days after the start of culture. Here, one unit of the enzyme activity is defined as an activity increasing 1 µmol of the Mn(III)-malonic acid complex over 1 minute. On the other hand, this activity was not observed in the supernatant of the target DNA-free strain OJI-1078 cultured under the same conditions.

Example 16

Construction of Expression Vector Containing *Coriolus hirsutus*-Derived Laccase Gene with *Coriolus hirsutus*-Derived *ras* Gene Promoter

[0082] The plasmid pCHRPMP obtained in Example 13 was digested with a restriction enzyme *Nco*I (Takara Shuzo Co., Ltd., Kyoto, Japan), blunt-ended with Klenow fragment, and digested with a restriction enzyme *Eco*RI to prepare the expression vector portion.

[0083] Next, a plasmid OJ-POG-E1 (Accession No. FERM BP-2793) containing a *Coriolus hirsutus*-derived laccase gene was amplified by PCR method using the following 2 primers:

Primer-1: 5'-CTCGAGGTTCCAGTCTCTG-3' (SEQ ID NO:9)
Primer-2: 5'-GAATTCCCGGGGACGTATACG-3' (SEQ ID NO:10)

[0084] The resulting PCR fragment was introduced into the TA-cloning vector to obtain pTALAC.

[0085] The obtained pTALAC was digested with restriction enzyme *Xho*I, blunt-ended with an modification enzyme Klenow fragment, digested with a restriction enzyme *Eco*RI to obtain the laccase structural gene portion, which was then introduced into the ras expression vector pCHRP treated above. The obtained plasmid was designated as pCHRPLAC (Fig. 8).

Example 17

Preparation of *Coriolus hirsutus* Transformant Highly Secreting and Producing Laccase

[0086] In transforming an arginine-requiring *Coriolus hirsutus* (OJI-1078 strain) with pCHRPLAC obtained in Example 16, a transformant pCHRPLAC/OJI-1078 was obtained by simultaneously introducing a *Coriolus hirsutus* OCT gene-carrying plasmid (pUCR1) as a selective marker (according to PEG method, electroporation or the like). Here, whether a DNA which can be subjected to transformation is in a circular or linear form, this transformation method could provide the transformant of interest. The conditions of transformation are as follows.

[0087] Two µg of the plasmid prepared in Example 8 was added to 100 µ l of the protoplast suspension having a concentration of approximately 10^6 cells/100 µ l in a circular or linear form, followed by addition of 0.2µg of pUCR1 as a selective marker then 30-minute ice-cooling.

[0088] Next, an equal volume of a PEG solution (50% PEG3400, 20mM MOPS (pH 6.4)) was added and the mixture was ice-cooled for 30 min. The ice-cooled mixture was mixed with a minimum soft agar medium (1% agar) containing 0.5M sucrose and leucine and inoculated onto a plate. The plate was cultured at 28°C for 4 days, whereby a transformant was obtained. From this transformant strain, a DNA is prepared, which was subjected to Southern hybridization in order to confirm that the target laccase-expressing plasmid had been introduced into the strain.

Example 18

Production of Laccase by Transformant

[0089] Five 50-mm^2 agar pieces containing the transformant strain obtained in Example 17 were inoculated into 50ml of a glucose-peptone liquid medium (30g/l glucose, 10g/l peptone, 1.5g/l KH_2PO_4, 0.5g/l $MgSO_4 \cdot 7H_2O$, 2mg/l thiamine HCl, 100mg/l $CuSO_4 \cdot 5H_2O$; adjusted to pH 5.0 with phosphoric acid) in a 500-mL conical flask and cultured at 28°C for 6 days with shaking. After 6 days, the obtained culture solution was centrifuged to obtain the supernatant.

[0090] The enzyme activity was measured by mixing 50 µl of 1M sodium acetate buffer (pH 4.0), 50 µ l of 5mM 2,2'-azino-bis(3-ethilbenzthiazoline-6-sulfonate) (ABTS)and 400 µ l of enzyme solution and recording the increase in 420-nm absorbance of ABTS oxide generated by the reaction over time. In the above supernatant, the enzyme activity was 40 units/ml on the 5th day after the start of culture. Here, one unit of the enzyme activity is defined as the quantity of an enzyme requiring to oxidize 1µmol of ABTS over 1 minute. On the other hand, the activity observed in the supernatant of the target DNA-free strain OJI-1078 cultured under the same conditions was only 5 units/ml.

INDUSTRIAL APPLICABILITY

[0091] The present invention provides a novel promoter region which functions in *Coriolus hirsutus* and enables the production of a lignin degrading enzyme, the mass production of the enzyme by genetic recombination having been considered to be difficult. Particularly, the transformant *Coriolus hirsutus* having an ability to highly degrade lignin according to the present inven- tion has a property of acting on lignin so as to degrade it into low-molecular-weight products, so it can be applied to various steps in paper and pulp production processes using a lignocellulose material (e.g., wood) as a raw material, that is, steps such as pulping, pulp bleaching, and waste water treatment. Moreover, in saccarification of wood, this transformant can also be applied to the field of a so-called cellulose biomass utilization for increasing the action of cellulase by degrading lignin in a treatment preceding the saccarification.

[0092] The respective sequences of SEQ ID NOS:1, 2 and 3 described herein are as follows:

SEQ ID NO:1:

ggatccgcta taccgaaagg ccgcgacgtc ctacacatgt cagtcaagag aacatgatgg 60
gcgtcatgtg gacagccgag ctgaggatgt attgcgcgag ggtgttcatc gagctgcgcg 120
tttccgagaa ctgctgtaga acggacgagg agcttcgaga ggggctgcga tggtgggaaa 180
tatctttccg gcggttttgg aagggccatt aagagcagag agatgcccgt ccaggacagg 240
gaaggtcggg tgcggactgt ggagcatccg cctgcgatcc tacatgagac cgggggcgag 300
acggacatct gggaaaggaa tgacatgcag ggaccgtgag aggatataca tgaatggtgg 360
tggtagacat accacgatgc tgggaacact atccgtgatg ttctcgcttg caccagaaag 420
gactgcaggt ggggagcaat cgcgccgaca ggtggcgtgc tatgcagaac cgcggcagtc 480
tcgacagcac cttacctctt ggacgtatca ctcacagtcc tctcggccct tcagatccaa 540
tcgatcccgt agtaacgtgc gtcatctagg agtggaaggt tggttgaccg acctgacaag 600
acataaccgg atgcccattc ggaaggctcg gggggccaag cgacctccgt gtatgatcgc 660
atgctatagc tcgacgtcgg gccgatagcg gcgcaaatca gagcaccgaa tgatgaagca 720
tctgagggaa gatcattgca tgagccatcc tgaacaggtt cgcaacgcgt ctgggaacga 780
gatgccatgc tgctacggtg atcctgatga agcacagccc gagatgcttg gtgctggacc 840
ccattggaag ctgctcgact tccttgtatg ataatcggtc tacatttctc gaccacagtt 900
gcgcatccgc ggtcagctga catcgaaggg gagcagtagt acagttggtg agctctcggg 960
ccttcgcggc gcattgccaa agacaccacc gaatattgcg aggccttgcg cagcgcgatg 1020
tggctaaata tgccagagca gctgtataag ggccctgtga ctcaccatgc gagaactgcg 1080
atatgtggct gtcagataat gcgatatacg agtcggagcg gaggcggaac tggggctggt 1140
agggactcta cttattgcgg taccggtcag aggatggcag cgttcagtga caagtcgcga 1200
agcgccgggc gcgagtattt ggctatgttt gcggcgcggt gtgttccaat agagggcgct 1260
tccacgtctt aattcccctg tcctcctcga cggatcacct ctcctccctc ccattcccgc 1320
ccttcaaata ccccccctcca cctcctcctc cgccatacag cc 1362

SEQ ID NO:2:

ggatccgcta taccgaaagg ccgcgacgtc ctacacatgt cagtcaagag aacatgatgg 60

gcgtcatgtg gacagccgag ctgaggatgt attgcgcgag ggtgttcatc gagctgcgcg 120
tttccgagaa ctgctgtaga acggacgagg agcttcgaga ggggctgcga tggtgggaaa 180
tatctttccg gcggttttgg aagggccatt aagagcagag agatgcccgt ccaggacagg 240
gaaggtcggg tgcggactgt ggagcatccg cctgcgatcc tacatgagac cgggggcgag 300
acggacatct gggaaaggaa tgacatgcag ggaccgtgag aggatataca tgaatggtgg 360
tggtagacat accacgatgc tgggaacact atccgtgatg ttctcgcttg caccagaaag 420
gactgcaggt ggggagcaat cgcgccgaca ggtggcgtgc tatgcagaac cgcggcagtc 480
tcgacagcac cttacctctt ggacgtatca ctcacagtcc tctcggcccc tcagatccaa 540
tcgatcccgt agtaacgtgc gtcatctagg agtggaaggt tggttgaccg acctgacaag 600
acataaccgg atgcccattc ggaaggctcg gggggccaag cgacctccgt gtatgatcgc 660
atgctatagc tcgacgtcgg gccgatagcg gcgcaaatca gagcaccgaa tgatgaagca 720
tctgagggaa gatcattgca tgagccatcc tgaacaggtt cgcaacgcgt ctgggaacga 780
gatgccatgc tgctacggtg atcctgatga agcacagccc gagatgcttg gtgctggacc 840
ccattggaag ctgctcgact tccttgtatg ataatcggtc tacatttctc gaccacagtt 900
gcgcatccgc ggtcagctga catcgaaggg gagcagtagt acagttggtg agctctcggg 960
ccttcgcggc gcattgccaa agacaccacc gaatattgcg aggccttgcg cagcgcgatg 1020
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SEQ ID NO:3:

Met Ser Arg Phe Leu Arg Glu Tyr Lys Leu Val Val Val Gly Gly Gly
1 5 10 15
Gly Val Gly Lys Ser Ala Leu Thr Ile Gln Phe Ile Gln Ser His Phe
20 25 30
Val Asp Glu Tyr Asp Pro Thr Ile Glu Asp Ser Tyr Arg Lys Gln Cys
35 40 45
Val Ile Asp Asp Glu Val Ala Leu Leu Asp Val Leu Asp Thr Ala Gly
50 55 60
Gln Glu Glu Tyr Gly Ala Met Arg Glu Gln Tyr Met Arg Thr Gly Glu
65 70 75 80
Gly Phe Leu Leu Val Tyr Ser Ile Thr Ser Arg Asn Ser Phe Glu Glu
85 90 95
Ile Ser Thr Phe His Gln Gln Ile Leu Arg Val Lys Asp Gln Asp Ser
100 105 110
Phe Pro Val Ile Val Val Ala Asn Lys Cys Asp Leu Glu Tyr Glu Arg
115 120 125
Gln Val Gly Met Asn Glu Gly Arg Asp Leu Ala Lys His Phe Gly Cys
130 135 140
Lys Phe Ile Glu Thr Ser Ala Lys Asn Arg Ile Asn Val Asp Glu Ala
145 150 155 160
Phe Ser Gln Leu Val Arg Glu Ile Arg Lys Tyr Asn Lys Glu Gln Gln
165 170 175
Thr Gly Arg Pro Gly Val Gln Pro Ser Ala Pro Ser Ala Pro Gly Val
180 185 190
Tyr Gly Asn Glu Lys Gly His Pro Asp Asp Gly Ala Gly Gly Cys Cys
195 200 205
Gly Cys Val Val Ala
210

[0093] Those skilled in the art will construe that various variations and modifications are possible within the scope of the invention described in the attached claims and the scope of its equivalence referring to the above descriptions. It

therefore should be understood that the present invention includes such variations and modifications too.

[0094] In addition, it is contemplated that all the publications and patent applications cited above, as well as two priority applications claimed in the present application, are incorporated herein by reference in their entireties.

Claims

1. A *Coriolus hirsutus* host cell transformed with a vector containing a basidiomycete-derived promoter region selected from the group consisting of *ras* and *priA* gene promoter regions from basidiomycetes.

2. A host cell according to Claim 1, wherein the *ras* gene promoter region is derived from *Coriolus hirsutus* or *Lentinus edodes.*

3. A *Coriolus hirsutus* host cell according to Claim 1, wherein the *priA* gene promoter region is derived from *Lentinus edodes*.

4. A *Coriolus hirsutus* host cell according to Claim 1, wherein the vector further comprises a gene encoding a useful polypeptide is transcirbably ligated at a site downstream of said promoter gene.

5. A *Coriolus hirsutus* host cell according to Claim 3, wherein said gene encoding a useful polypeptide is a gene coding for a lignin degrading enzyme such as manganese peroxidase, lignin peroxidase, or laccase gene.

6. A process for producing a useful polypeptide comprising culturing the *Coriolus hirsutus* host cell defined in Claim 1 in a medium and recovering the formed useful polypeptide.

7. A process according to Claim 6, wherein the useful polypeptide is a lignin degrading enzyme such as manganese peroxidase, lignin peroxidase, or laccase.

8. An isolated DNA fragment containing a *Coriolus hirsutus*-derived *ras* gene promoter region.

9. An isolated DNA fragment according to Claim 8, wherein the DNA fragment has a nucleotide sequence shown in SEQ ID NO:1 or a sequence that hybridizes to a sequence complementary to the said nucleotide sequence under stringent conditions and has a promoter activity.

10. A recombinant DNA containing a gene encoding a useful polypeptide and the DNA fragment defined in Claim 8, the gene being transcribably linked to the DNA fragment.

11. A recombinant DNA according to Claim 10, wherein the gene encoding a useful polypeptide is a gene coding for a lignin degrading enzyme such as manganese peroxidase, lignin peroxidase, or laccase.

12. A DNA which contains *Coriolus hirsutus*-derived *ras* gene promoter sequence and *ras* gene sequence and has a nucleotide sequence shown in SEQ ID NO:2.

13. A vector containing the DNA fragment defined in Claim 8 or the recombinant DNA defined in Claim 10.

14. A host cell transformed with the vector defined in Claim 13.

15. A host cell according to Claim 14, wherein the host is a basidiomycete.

16. A host cell according to Claim 15, wherein the basidiomycete is *Coriolus hirsutus* .

17. A process for producing a useful polypeptide comprising culturing the host cell defined in Claim 13 in a medium and recovering the formed useful polypeptide.

18. A process according to Claim 17, wherein the useful polypeptide is a lignin degrading enzyme such as manganese peroxidase, lignin peroxidase, or laccase.

FIG. 1

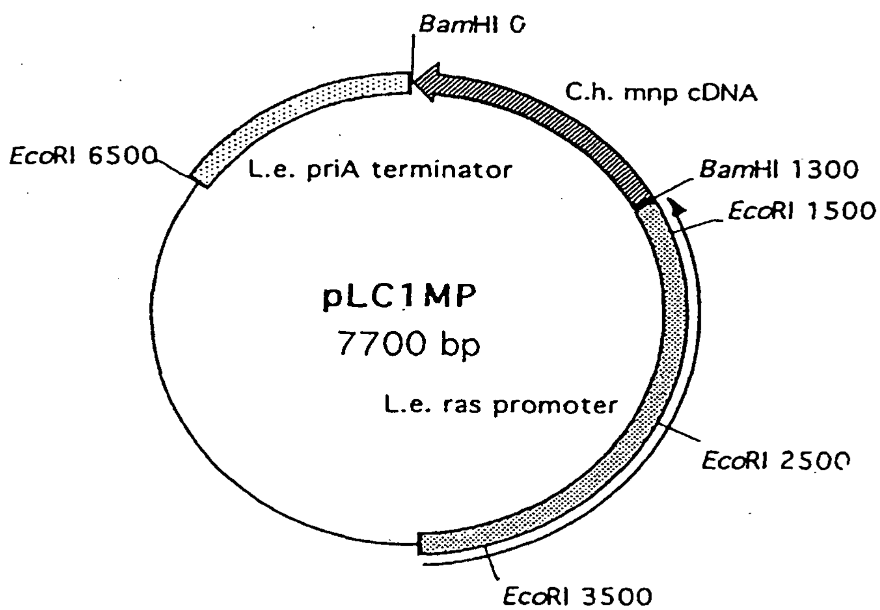
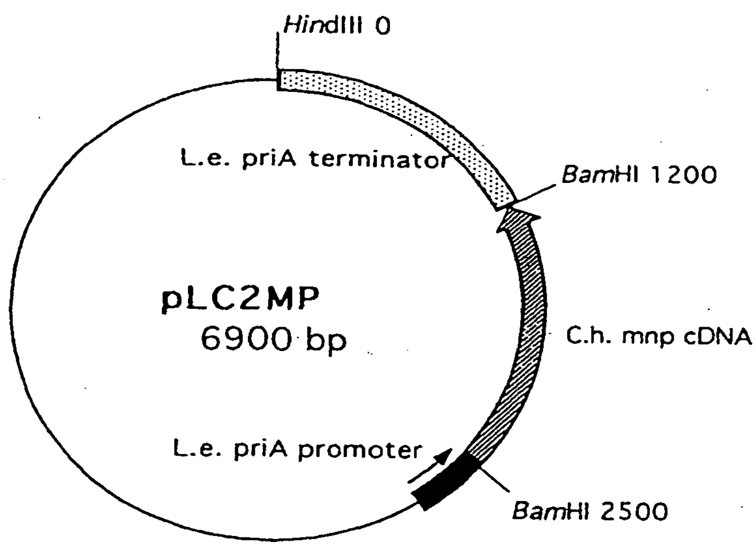

BamHI 0
C.h. mnp cDNA
EcoRI 6500
L.e. priA terminator
BamHI 1300
EcoRI 1500
pLC1MP
7700 bp
L.e. ras promoter
EcoRI 2500
EcoRI 3500

FIG.2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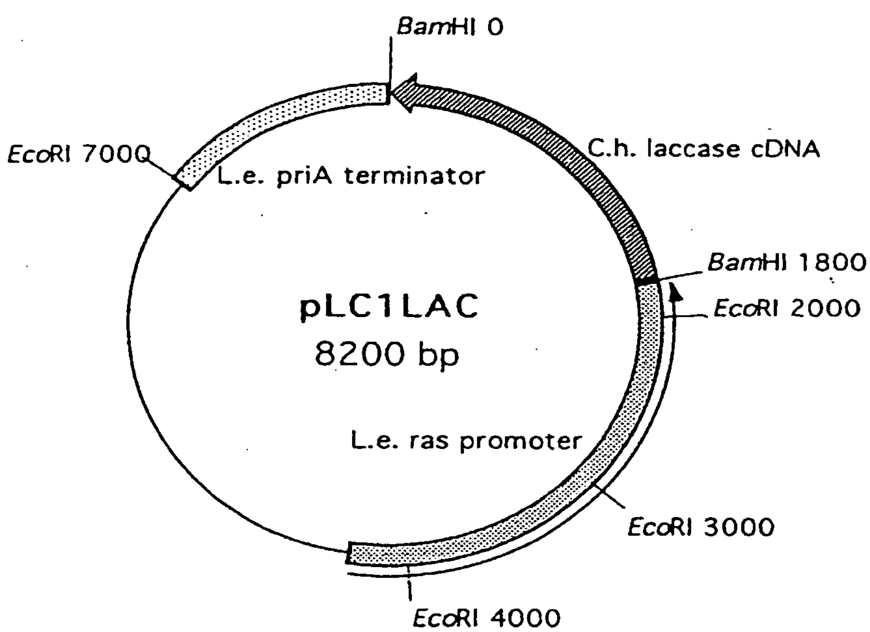
L.e. priA terminator
BamHI 1200
pLC2MP
6900 bp
C.h. mnp cDNA
L.e. priA promoter
BamHI 2500

FIG.3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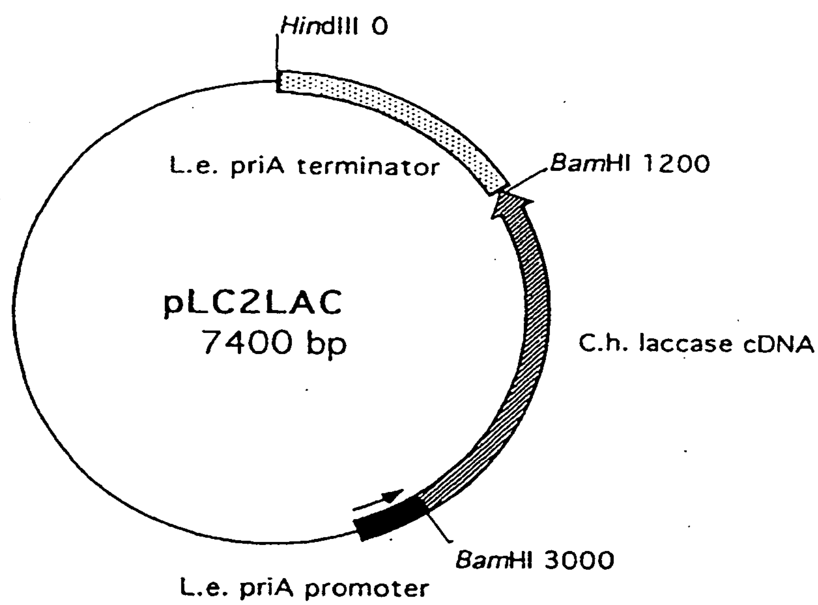
EcoRI 7000
L.e. priA terminator
C.h. laccase cDNA
BamHI 1800
EcoRI 2000
pLC1LAC
8200 bp
L.e. ras promoter
EcoRI 3000
EcoRI 4000

FIG.4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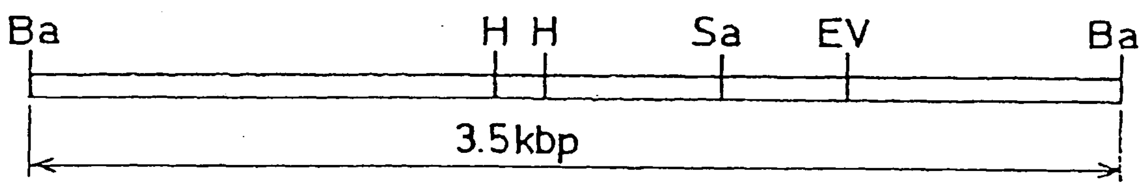
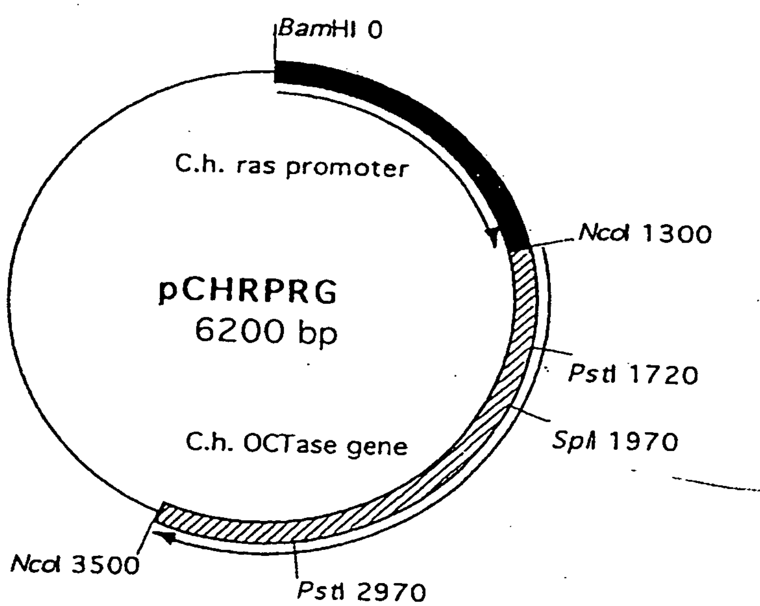
pLC2LAC
7400 bp
C.h. laccase cDNA
BamHI 3000
L.e. priA promoter

FIG.5



Ba: BamHI, HindIII. Sa: SalI, EV: EcoRV

FIG.6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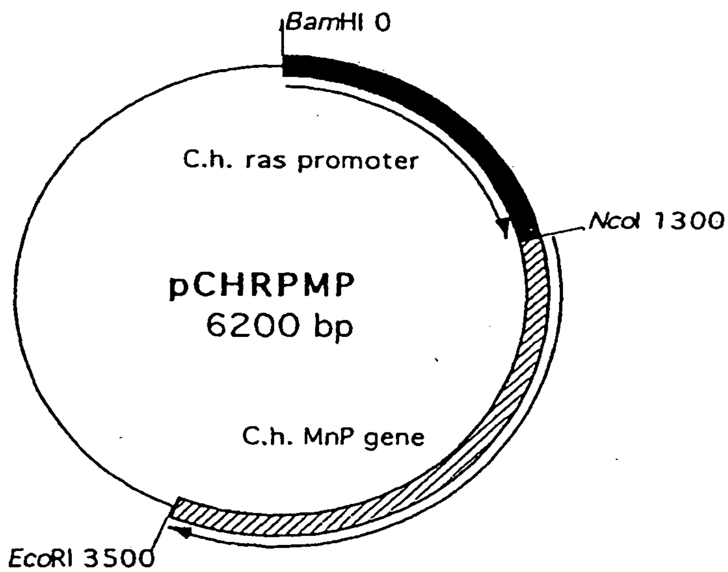
NcoI 1300
pCHRPRG
6200 bp
PstI 1720
SplI 1970
C.h. OCTase gene
NcoI 3500
PstI 2970

FIG.7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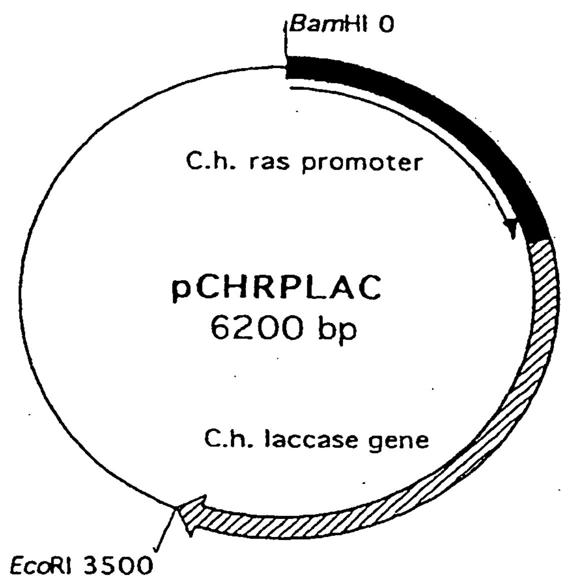
6200 bp
C.h. MnP gene
EcoRI 3500

FIG.8


BamHI 0
C.h. ras promoter
pCHRPLAC
6200 bp
C.h. laccase gene
EcoRI 3500